PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/17, A61K 48/00, 35/34, A61P 5/48 | A1

(11) International Publication Number: **WO 00/31267**

(43) International Publication Date: 2 June 2000 (02.06.00)

(21) International Application Number: PCT/EP99/09132

(22) International Filing Date: 19 November 1999 (19.11.99)

(30) Priority Data:
60/109,181 20 November 1998 (20.11.98) US

(71) Applicant *(for all designated States except US)*: THE AUTONOMOUS UNIVERSITY OF BARCELONA [ES/ES]; Edifici V, E–08193 Bellaterra (ES).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: BOSCH, Fatima [ES/ES]; The Autonomous University of Barcelona, Edifici V, E–08193 Bellaterra (ES).

(74) Agents: HUTCHINSON, Glenn; Dibb Lupton Alsop, Fountain Precinct, Balm Green, Sheffield S1 1RZ (GB) et al.

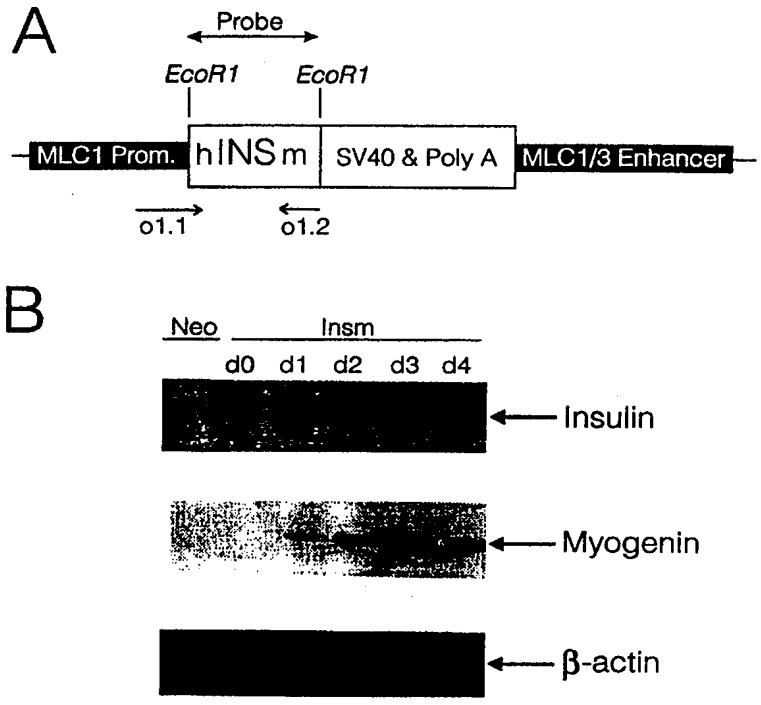
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: INSULIN PRODUCTION BY ENGINEERED MUSCLE CELLS

(57) Abstract

A method of treating diabetes mellitus in a subject comprising either direct incorporation of a gene encoding insulin in the muscle of the subject or of transfecting a muscle cell line with a gene encoding insulin and introducing the engineered muscle cell into the subject.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

Insulin Production by Engineered Muscle Cells

Background of the Invention

Insulin –dependent diabetes mellitus (Type I diabetes) results from autoimmune destruction of the pancreatic islet ß-cells, leading to long term insulin deficiency, hyperglycemia and the development of secondary microvascular and neurological complications (Rudderman, et al., FASEB J., 1992, 6, 2905-2914). The risk of complications increases with the degree of hyperglycemia. Intensive insulin therapy can delay the onset and slow the progression of microvascular complications (The Diabetes Control and Complication Trial Research Group, N Engl J Med., 1993, 329, 977-986). However, this kind of treatment cannot be easily implemented for all diabetic patients, especially in the very young and very old.

Modern insulin regimes aim to mimic physiological insulin profiles, with relatively constant low background levels on which are superimposed post-prandial peaks of insulin injections. Most patients are treated with subcutaneous injection of insulin preparations that have rapid absorption and short action or suspensions of insulin that give intermediate or long acting profiles. Also the mixture of soluble insulin with the long acting lente insulin reduces the availability of the short-acting componenet (Heine, Bio. Med J., 1985, 290, 204-205). Furthermore one of the major difficulties with the delayed-action insulins is the variable absorption from subcutaneous tissue (Binder, Diabetes Care, 1984, 7, 188-199). Thus, a smooth background level of insulin cannot be easily attained to prevent fluxes in plasma glucose especially in the post-prandial and interprandial period.

An alternative to ultra-long acting insulin preparations is the production of engineered cells that could result in a sustained and constant low level secretion of basal insulin in the host.

Restoration of endogenous insulin secretion via transplantation of pancreatic islets have been attempted with mixed results. The reason for this is that the underlying autoimmunity towards the ß-islet cells will continue to destroy the transplanted islets. The use of heterologously derived ß-cell lines which allow some level of regulated production of insulin are end in a loss of glucose responsiveness. (Efrat, Diabetes, 1993, 42, 901-907; Ferber, J Biol Chem., 1994, 269, 11523-11529). Nevertheless, a RIN 1046-38 cell line showing increased insulin content and improved range of response to glucose has recently been obtained by stable transfection with a combination of genes encoding the human insulin, GLUT2 and glucokinase (Clark, Diabetes, 1997, 46, 958-967; Hohmeier,

Diabetes, 1997, 46, 968-977). These cells could be encapsulated for transplantation. However, because this a pancreatic tumor cell line, they undergo rapid cell division, thus increasing oxygen and nutrient demand and produce metabolic by products that affect their survival in the capsule and also that of the host. An alternative strategy is to engineer a non-dividing cell line that could allowed a continuous production of basal insulin to meet the needs during the postprandial and interprandial peroid to be used in conjunction with conventional meal associated injection of currently available insulin preparations.

In contrast to ß-cells, myoblast cell lines like C2C12 have the advantage of a rapidly dividing in the myoblast stage and the potential for differentiating into non-dividing myotubes, a crucial feature for viability after transplantation. These cells can be genetically engineered, and are amenable to production in large quantities in the myoblast state and then subjected to differentiation to allow production of the recombinant proteins with the use of a suitable promoter, and kept alive for long term delivery of the recombinant protein. If primary myoblast were isolated from a host, and then genetically engineered to produce, for example insulin, they can be stably returned to the host by simple injection into the muscle, where they will fuse with the other muscle fibers (Barr and Leiden, Science, 1991, 254, 1507-1509; Dai, Proc Natl Acad Sci USA., 1992, 89, 10892-10895, Dhawan, Science, 1991, 254, 1509-1512) or upon encapsulation (Deglon, Human gene Therapy, 1996, 7, 2135-2146; Rinsch, Human Gene Therapy, 1997, 8, 1881-1889) allow for the secreted products to enter the circulation.

Summary of the Invention

An aspect of the invention features a process of treating diabetes in a subject comprising the step of administering to the subject a DNA segment or gene encoding for insulin, the gene being under the control of a promoter sequence in which the promoter sequence is operably linked to the to the insulin gene and is effective for the expression of a therapeutically effect amount of insulin in the diabetic subject. It is expected that the expression of insulin in diabetic subject will help to obtain a tighter control of the subjects glucose level in conjunction with conventional exogenous insulin treatment administered with meals in the case of type 1 diabetes mellitus or in conjunction with other antidiabetic therapies (e.g. sulfonylureas, biguanides, α-glucosidase inhibitors, glitazones, and other insulin secretagogues and insulin-enhancing agents) for type 2 diabetes mellitus. The term "therapeutically effective amounts means an amount of the expressed insulin which is sufficient to effect glucose uptake into cells, tissues and organs of a diabetic subject, and can be determined without undue experimentation.

As used here in, the term "insulin" refers to a mature, active, polypeptide comprising substantially of the amino acid sequence of the natural hormone insulin. As an example of such an insulin is human insulin or porcine insulin or Lispro insulin. The insulin coding sequence of the DNA segment can be the same or substantially the same as the coding sequence of the endogenous insulin coding sequence as long as it encodes a functional insulin protein. As such the DNA segment can also be the same or substantially the same as the insulin gene of a non-human species as long as it encodes a functional insulin protein.

The insulin gene in the DNA segment is preferably under the control of a promoter sequence different from the promoter sequence controlling the endogenous coding sequence, e.g. a promoter sequence which remains activated or induced during diabetic conditions in the patient. Examples of such promoter sequences include the muscle specific myosin light chain (MLC) promoters – MLC1, MLC2, MLC3; creatine kinase, and myoD.

In a second aspect, the present invention comprised of a transformed myoblast or muscle cell capable of producing mature insulin. As used here in, the term "transformed cell" refers to a cell that have been transfected with either homologous (same species) or heterologous (different species) gene coding for the insulin. The term "muscle cell" refers to any cells human or non-human of muscle lineage or has the propensity to form a muscle cell, e.g. precursor muscle cell or myoblast. As examples of muscle cells are the mouse lines C2C12, C3H/10T1/2, rat L6 and L8, human HISM.

In one embodiment, the DNA segment is introduced to the diabetic patients in muscle or myoblast cells, wherein the cells are treated in vitro to incorporate therein the DNA fragment and, as a result, the cells express in vivo in the diabetic patient a therapeutically effective amout of insulin. The DNA segment can be introduced into the cell by standard gene transfection methods, e.g. calcium phosphate precipitation or by a viral vector, e.g. adenoviral vector. The cells may be introduced to the host by standard transplantation techniques, or in a neoorgan, or in a matrix, e.g. microencapsulated in sodium alginate, or contained within a immunoprotected cell factory.

In another embodiment, the DNA segment is directly introduced into the muscle of the diabetic patient, e.g. not contained within a cell. The DNA segment can be introduced in a vector. Examples of suitable vectors include viral vectors (e.g. retroviral vectors, adenoviral vectors, adeno-associated viral vectors, sindbis viral vectors, and herpes viral vector), plasmids, cosmids, and yeast artificial chromosomes. The DNA segment can also

be introduced as infectious particles, e.g., DNA-ligand conjugates, calcium phosphate precipitates, and liposomes.

Brief Description of the Figures

Figure 1. Expression of human insulin in C2C12 differentiated myotubes. (A) Schematic representation of the MLC1/Insm chimeric gene. (B) Northern blot analysis of MLC1/Insm chimeric gene expression in total RNA obtained from control C2C12neo and C2c12Insm from day 0 to day 4 of differentiation along with the reference level of myogenin, and ß-actin expression. (Gros, L., et al., Human Gene Therapy, 10:1207-1217 (1999)

Figure 2. Detection of human insulin by immunofluorescence analysis of C2C12 differentiated myotubes stably transfected with MLC1/Insm chimeric gene. (a) C2C12, (b) C2C12neo, (c) and (d) C1C12Insm cells . Magnification 400X in (a),(b),(d), and 800X in (c).

Figure 3. HPLC analysis of insulin immunorecative products in cells extracts and in culture media of C1C12Insm cells. After 3 days of differentiation. Culture media (A) and cell extracts (B) were subject to HPLC fractionation and RIA analysis. Arrows indicate the position of the mature insulin and proinsulin peaks according to the elution time relative to the standards. Standard medium containing 5 X 10^{-4} M porcine insulin and 5 X 10^{-5} M human recombinant insulin (o); culture medium and cell extracts from C2C12neo (□) and C2C12Insm (•).

Figure 4. Insulin production by differentiated C2C12Insm myoblast cells. (A) Myoblast cell were differentiated and at indicated times, aliquots of the culture medium were obtained and analyzed for immunoreactive insulin by RIA, C2C12neo (o); C2C12Insm (•). (B) Myoblast cell were maintained differentiated for 42 days. At the indicated days C2C12Insm (•) cells were incubates for 2 hrs in serum free medium and immunoreactive insulin in the medium was maesured. Results are expressed mean ± SEM of three different experiments, each performed in triplicates.

Figure 5. Effect of insulin gene expression on the rate of glucose uptake and lactate production by C2C12Insm differentiated myotubes. Cells were differentiated for 3 days and then cultured overnight in serum-free medium. At the indicated times, aliquots of the medium fromC2C12neo (o) and C2C12Insm (•) cells were obtained. Glucose concentrations (A) and lactate production (B) were determined. Results are the mean ± SEM of three different experiments, each performed in triplicates.

Figure 6 Effect of insulin produced buy C2C12Insm cells and exogenous insulin on PEPCK gene expression in FTO-2B hepatoma cells. Total RNA from the FTO-2B cells

were analyzed by Northern blot analysis using a PEPCK cDNA as a probe. (A) A representative Northern blot is shown; (B) Densitometric analysis of autoradiograms was performedand results expressed a % of the basal PEPCK gene expression. Results are the mean ± SEM of three different experiments.

Figure 7. Effect of transplantation of C2C12Insm cells into skeletal muscle of diabetic mice.`On week after the end of STZ treatment, mice were transplanted with C2C12 (o) or C2C12Insm (•) myoblast cells . Insulin (A) and glucose (B) concentrations were measured during the three weeks after transplantation. Plasma insulin concentration of healthy C3H mice is indicated in (A) (□).

Detailed Description of the Invention

The method of making and using DNA segments to practice the therapeutic process of this invention are well within the ability of a person of ordinary skill in the art. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Also all publications cited herein are incorporated by reference.

The therapeutic process of the invention allows for the production, at higher levels than pretreatment of insulin in a diabetic patient. The production of insulin in the diabetic patient results in an increase in the target tissue level and or circulating levels of the hormone that results in an increased uptake of glucose into cells within the patient as evident in the increased disposal of glucose from the blood stream after a meal.

What is meant by "DNA segment" herein is any exogenous DNA construct which includes a sequence encoding for a proinsulin polypeptide (Bondy & Rosenberg, Metabolic Control and Disease, Eigth Ed., 1980, pp. 284-287, WB Saunders) that may be processed to a functional insulin (Bondy & Rosenberg, Metabolic Control and Disease, Eigth Ed., 1980, pp. 284-287, WB Saunders), and the insulin is expressed by cells into which the DNA segment is introduced. The DNA segment can be introduced into both somatic muscle cells of a patient, or may be introduced ex vivo into the muscle cells or precursor cells with the potential to differentiate into a muscle cells, myoblasts. The muscles cells may or may not be derived from the patient treated, and may be derived from a non-human host species, e.g. mice, rat, pig. The DNA segment, therefore may or may not be an integral part of the patient's chromosome, and if the DNA segment is integrated into a chromosome, it may or may not be located at the same site as its corresponding endogenous gene sequence.

The DNA segment used to practice the therapeutic process includes an insulin gene or its complementary DNA ("cDNA") that is substantially similar to the mammalian

insulin gene from various species, e.g., mice, rat, pig, bovine, ovine and human, lispro insulin. Preferably, the insulin gene is an engineered proinsulin gene that will code for a proinsulin precursor peolypeptide that will be constitutively processed in muscle cells to a mature, active insulin (Vollenweider, et al., J Biol Chem., 1992, 267, 1429-14636; Yanagita, M., et al., FEBS Lett., 1992, 311, 55-59; Groskreutz, D. J., et al., J Biol Chem., 1994, 269, 6241-6245).

The expression of the DNA segment is driven by a promoter which is expressed during diabetes conditions. Examples of suitable promoters includes the strong muscle specific constitutive promoters, e.g. Myosin light chain promoters (Lee, et al, J Biol Chem., 1992, 267, 15875-15885; Greishammer, et al, Cell, 1992, 69, 79-93), Muscle creatine kinase promoter (Yi, T.M. et al. al., Nuc Acid Res, 1991, 19: 3027-3033; Morlick, R.A. et al. al., Mol. Cell Biol, 1989, 9:2396-2413), Myogenin promoter (Cheng, T.C. et al. al., Science, 1993 261:215-218), MyoD promoter (Tapscott, S.J., et al. al., Mol. Cell Biol., 1992, 12: 4994-5003). The promoter is comprised of a cis-acting DNA sequence capable of directing the transcription of a gene in the appropriate environment, tissue, context.

Examples of cells targeted for the production of insulin includes primary muscle cells isolated from the patients or a different patient (Salminer, et al., Human gene Therapy, 1991, 2: 15-26;) are the mouse lines C2C12 (ATCC# CRL1772), C3H/10T1/2 (ATCC# CCL226), rat L6 (ATCC# CRL1458), L8 (ATCC# CRL 1769), human HISM (ATCC# CRL1692).

To practice the therapeutic process of this invention, one can use vectors (both viral and non-viral, e.g. plasmid, cosmid, and yeast or bacterial artificial chromosomes and gene delivery systems available for either in vitro expression into cells utilized in ex vivo implantation or direct in vivo delivery of insulin into muscle cells or tissue of a patient. Detailed guidance is provided below:

Viral Vectors for Delivery of an Insulin Gene

Viral vectors can be used for delivery of an insulin gene. Examples of viral vectors include recombinant retroviral vectors, recombinant adenoviral vectors, recombinant adeno-associated viral vectors, sindbis viral vectors and recombinant herpes viral vectors.

(a) Recombinant retrovirus vectors

The genome of a conventional recombinant retroviral vector is comprised of long terminal repeat ("LTR") sequences on both ends that serve a viral promoter/enhancer and transcription initiation site, a Psi site that serve as virion packaging signal and a selectable marker gene, for example a neomycin resistance gene. Examples of such vectors include pZIP-NeoSV (Cepko, et al., Cell, 1984, 1953-1062). The insulin gene can be cloned into a

suitable cloning site in the retroviral genome. Expression is under the transcriptional control of the retroviral LTR. The vector will drive the constitutive expression of insulin in myoblast or muscle cell. The level of expression is dictated by the promoter strength of the LTR. The tissue selectivity is generally determined by the origin of the viral genome (for example, sarcoma virus/leukemia virus/mammary tumor virus).

Specific modifications in the sequence of the LTRs to improve the level of expression of the cloned gene have been described (Hilberg, et al., PNAS USA, 1987, 84, 5232-5236; Holland, et al, PNAS USA, 1987, 84, 8662-8666, Valerio, et al., Gene, 1989, 84, 419-427). The insulin gene can also be cloned into the vector linked to an internal promoter can confer tissue specificity to the control on gene expression (Lai, et al., PNAS USA, 1989, 86, 10006-10010; Scharfmann, et al., PNAS USA, 1991, 88, 4626-4630). Examples of internal promoter may be a general, strong constitutive promoter, for example the B-Actin promoter (Kawamoto, et al., MCB, 1988, 8, 267-272; Morishita, et al., BBA, 1991, 1090, 216-222; Lai, et al., PNAS USA, 1989, 86, 10006-10010), the muscle specific myosin light chain 2 (Lee, et al., J Biol Chem., 1992, 267, 15875-15885; Shen, et al., MCB, 1991, 11, 1676-1685; Lee, et al., MCB, 1994, 14, 1220-1229),myosin light chain 1/3 (Grieshammer, et al., Cell, 1992, 69, 79-93; Donoghue, et al., Gene Dev., 1988, 2, 1779-1790), alpha-myosin heavy chain (Molkentin, et al., J Biol Chem., 1993, 268, 2602-2609) promoters. Example of such retroviral vectors include, vLPGKSN (Valera, et al., Eur J Biochem., 1994, 222, 533-539), mLBSN (Ferrari, et al., Human Gene Therapy, 1995, 6, 733-742).

The glucokinase gene is cloned into the vector downstream from the internal promoter, generally as an expression cassette (Crystal,R.G., Science, 1995, 270, 404-410).

The recombinant retroviruses capable of transducing the insulin gene into cells, in vivo, ex vivo, or in vitro and directing the synthesis of the insulin polypeptide in the infected cells, are produced by transfecting the recombinant retroviral genome(s) into a suitable (helper-virus free) amphotropic packaging cell line. A number of virus packaging cell lines are now available, PA317, Psi CRIP (Cornetta, et al., Human Gene Therapy, 1991, 2, 5-14, Miller & Buttimore, MCB, 1986, 6, 2895-2902; Cone & Mulligan, Proc Natl Acad Sci USA., 1984, 81, 6349-6353). The transfect virus packaging cell lines will package and produce recombinant retroviruses, shedding them into the tissue culture media. The retroviruses are then harvested and recovered from the culture media by centrifugation as previously described (Compere,et al., MCB, 1989, 9, 6-14). The viruses

may be resuspended in a suitable buffer, for example, 10 mM HEPES (Sigma, St. Louis, MO) and stored at -70°C or under liquid nitrogen.

(b) Recombinant Adenovirus vectors

Adenovirus vectors can be used for transducing an insulin expression cassette into cells (Berkner, et al., BioTechniques, 1988, 6, 616-629). Constitutive high levels of expression of the transduced gene products can be achieved. These vectors have the inherent advantage over the retroviral vectors in that they can infect replicating and not replicating cell, making them suitable vectors for somatic gene therapy (Mulligan, R.C., Science, 1993, 260,926-932).

Replication defective adenoviruses lacking the E1 region of the genome have been developed which will accomodate the insertion of 7.5 kilobases of foreign DNA (Crystal, R.G., Science, 1995, 270, 404-410; Logan & Shenk, PNAS USA, 1984, 81, 3655-3659; Freidman, et al., MCB, 1986, 6, 3791-3797; Levrero, et al, gene, 1991, 101, 195-202; Imler, et al., Human Gene Therapy, 1995, 6, 71-721). These replication defective recombinant adenoviruses can be propagated by transfecting the genome into cells engineered to express the E1 genes (Jones & Shenk, Cell, 1979,16, 683; Berkner, et al., BioTechniques, 1988, 6, 616-629). This system allows the production of adenovirus particles at high titer (up to 10^{13}/ml) which greatly enhance infection efficiency by enabling a higher multiplicity od infection (Crystal, R.G., Science, 1995, 270, 404-410).

Strategies for generating Adenoviral recombinants have been described (Berkner, et al., BioTechniques, 1988, 6, 616-629). An example is the use of the plasmid pMLP6 (Logan & Shenk, PNAS USA, 1984, 81, 3655-3659) which carry the Adenovirus 5 genome with the E1 region deleted. Digestion with the restriction endonucleases *Bgl*II and *Rsa*I will produce a linearized plasmid that retains only the left most 194 bp of the Adeno-5 genome. An expression cassette containing a regulatory and tissue specific promoter region for example the MLC-1 promoter linked to a DNA fragment encoding the human insulin with compatible 3' and 5' ends (modified by appropriate linker ligations and then subjecting appropriate restriction endonuclease diegstion as described in Maniatis, et al., Molecular Cloning-A Laboratory Manual, CSHL, CSH, 1989) can be cloned into the Adeno-5 plasmid. The entire recombinant Adenovirus genome is then generated by mixing the linearized Adeno-5-MLC-1-human Insulin plasmid with a subgenomic fragment of Adenovirus DNA representing the 3.85-100 map units (prepared by digesting the In340 viral genome with *Cla*I or *Xba*I) (N.E. Biolabs, Beverly, MA) (Berkner, et al., BioTechniques, 1988, 6, 616-629). The DNAs are then transfected into 293 cells (Graham, et al., J Gen Virol, 1977, 36, 59-72) essentially as described (Berkner & Sharp,

Nuc Acid Res, 1983, 11, 6003-6020). Intermolecular recombination across appropriate segments of the plasmid and the subgenomic fragment of Adenoviral DNA will result in the production of replication defective recombinant adenoviral genomes carrying the MLC1-human Insulin chimeric gene. The recombinant genomes will emerge from the 293 cell lines as packaged viral particles shed into the medium. Modifications of this design that result in high level expression vectors have been developed (Berkner, et al., BioTechniques, 1988, 6, 616-629) by incorporating regions of the major late promoter and the tripartite leader elements (Berkner & Sharp, Nuc Acid Res, 1983, 11, 6003-6020; Logan & Shenk, PNAS USA, 1984, 81, 3655-3659) in the vector construction .

The use of recombinant adenoviruses have been successfully use to delivery genes into cells of animals (Katkin, et al, Human Gene Therapy, 1995, 6, 985-995). The feasibility for transducing genes associated with glycogen metabolism using adenovirus-mediated transfer in primary rat myoblast in culture, has been recently described (Baque, et al., Biochem J., 1994, 304 (Pt 3), 1009-1014; Gomez-Foix, et al., J Biol Chem., 1992, 267, 25129-25134).

(c) Recombinant Adeno-associated viruses:

Adeno-associated virus ("AAV") can also be used as a vector for transducing the insulin gene expression cassette (Fisher, K.L., et al., Nature Medicine, 1997, 3, 306-312). AAV offers the advantage in that it has not been implicated in the etiology of any disease and its site specific integration on human chromosome 19 has not been shown to interferes host gene expression or promote gene rearrangements (Kotin, et al., PNAS USA., 1990, 87, 2211-2215; Samulski, et al., EMBO J., 1991, 10, 3941-1950). Like the adenoviruses, AAV is capable of infecting postmitotic cells making it a suitable vector for delivery of genes to somatic cells.

The AAV genome contains two genes, *rep* and *cap*, and inverted terminal repeats (ITR) sequences (Hermonat, et al., J Virol., 1984, 51, 329-339). Recombinant AAV vectors are constructed by replacing the rep gene, the cap gene, or both with the insulin gene expression cassette (Hermonat, et al., PNAS USA., 1984, 81, 6466-6470). The sole sequence needed for AAV vector integration is the terminal 145 base ITR ((Muzyczka, N., Curr Top Microbiol Immunol., 1992, 158(97), 97-129). Such vectors are available in the plasmid form (Tratschin, et al., MCB., 1985, 5, 3251-3260; Lebkowski, et al., MCB., 1988, 8, 3988-3996; McLaughlin, et al., J Virol., 1988, 62, 1963-1973).

The recombinant AAV genomes can be packaged into AAV particles by co-transfection of the vector plasmid and a second packaging plasmid carrying the *rep* and *cap* genes into an adenovirus-infected cell. Such particles have been shown to efficiently

transduce heterologous genes into mammalian cell lines, including muscle cells (Tratschin, et al., MCB., 1985, 5, 3251-3260; Lebkowski, et al., MCB., 1988, 8, 3988-3996; McLaughlin, et al., J Virol., 1988, 62, 1963-1973; Flotte, et al., Am J Respir Cell Mol Biol., 1992, 7, 349-356; Fisher, K.L., et al., Nature Medicine, 1997, 3, 306-312).

In addition to using an expression cassette, high levels of expression of genes linked directly to the endogenous AAV p40 promoter has been demonstrated (Wondisford, et al., Mol Endocrinol, 1988, 2, 32-39).

(d) Recombinant Herpes virus vectors:

Herpes virus vectors ("HSV") constitute a unique system for the delivery of genes into cells of neuronal lineage (Anderson, et al., Cell Mol Neurobiol., 1993, 13, 503-515). Herpes simplex virus(HSV)-derived vectors will infect postmitotic neurons, produce an established latent infection in some cell types, making it a suitable system for somatic gene therapy (Leib & Olivo., BioEssays., 1993, 15, 547-554).

Strategies for the generation of HSV vectors and recombinant viruses suitable, for example for the transduction of the insulin gene, has been described (Leib & Olivo., BioEssays., 1993, 15, 547-554). The general method extensively used for mutagenizing endogenous viral genes (Post & Roizman, Cell, 1981, 25, 227-232) may be applied for the introduction of exogenous genes like insulin gene into the HSV genome.

The insulin expression cassette is cloned into a plasmid containing a portion of the HSV genome such that at least 300 bp flank the 5'- and 3' ends of the cassette (Breakfield & Deluca., New Biol ., 1991, 3, 203-218; Efstathiou & Minson,., Brit Med Bull., 1995, 51, 45-55). The plasmid is transfected into permissive cells in culture along with the full length HSV DNA (Geller., et al., PNAS USA., 1990, 87, 8950-8954). Homologous recombination and DNA replication will result in the generation of recombinant HSV genomes that are packaged into novel virus particles by the cell. Through several round of plaque purification, a recombinant virus carrying the insulin expression cassette may be identified for large scale production.

Defective HSV vectors have benn successfully used to transfer exogenous genes into neuronal cells in vitro and in vivo (Geller & Freese., PNAS USA., 1990, 87, 1149-1153; Geller & Breakfield., Science, 1988, 241, 1667-1669; reviewed in Efstathiou & Minson,., Brit Med Bull., 1995, 51, 45-55). A variety of constitutive promoters have been used including the lytic cycle HSV promoters, the RSV LTR, the HCMV IE promoters and the neurofilament and PGK promoters for transient expression. Long term expression have been obtained using the Moloney murine leukemia virus LTR, HSV LAT promoter, HCMV IE promoter fused to the LAT promoter elements,and the neuro specific enolase

promoter. These vectors have been also reported to be useful for transduction of genes into cells of non-neuronal origin (Efstathiou & Minson,., Brit Med Bull., 1995, 51, 45-55; Miyanohara, et al., New Biol., 1992, 4, 238-246).

(e) Sindbis virus vectors

Sindbis virus-based vectors are intended as self-amplifying systems to enhance expression of exogenous genes in mammalian cells (Herweijer, et al., Human Gene Therapy, 1995, 6, 1161-1167).

The 5'-two thirds of the Sindbis virus genome encodes the nonstructural genes needed for replication of the viral genome. The 3'-third of the genome encode the structural proteins (Strauss, et al., Virology, 1984, 133, 92-110; Strauss & Strauss, Microbiol. Rev., 1994, 58, 491-562). In the sindbis virus self-amplifying expression systems, the subgenomic sequence coding for the structural proteins are replaced by the expression cassette of the transgene, for example, the insulin gene (Huang, et al., Virus Genes, 1989, 3, 85-91; Bredenbeek, et al., J Virol., 1993, 67, 6439-6446).

Generally the RNA genome of the recombinant sindbis virus is generated by placing the entire genome under the control of the bacteriophage T7 or SP6 peomoters to enable transcription of the (+) strand RNA in vitro (Herweijer, et al., Human Gene Therapy, 1995, 6, 1161-1167). The resultant RNA genomes are then used to transfect target cells (Xiong, et al., Science, 1989, 243, 1188-1191). Infectious viruses are produced by infecting with a helper virus (Bredenbeek, et al., J Virol., 1993, 67, 6439-6446). Modifications of this design using the Rous sarcoma virus LTR to direct the transcription of the non-structural genes have been described (Herweijer, et al., Human Gene Therapy, 1995, 6, 1161-1167).

To generated a recombinant Sindbis virus vector, the luciferase gene cloned into the unique *Xba*I site in the vector pSin-Lux (Herweijer, et al., Human Gene Therapy, 1995, 6, 1161-1167) is replaced by the insulin cDNA or an expression cassette encoding glucokinase upon appropriate restriction endonuclease modifications (Sambrook, et al., Molecular Cloning- A Laboratory Manual, CSHL, CSH, 1989).

Sindbis virus vectors have been successfully used to transduce foreign genes into primary rat myoblasts (Herweijer, et al., Human Gene Therapy, 1995, 6, 1161-1167).

<u>In vivo Delivery of an Insulin gene by viral infection</u>

Viral vectors can be used to deliver the insulin coding sequence into the cells tissues of diabetic patients by in vivo infection. In in vivo infection, the recombinant viral vector is administered to the organism in order to result in a tissue specific infection of the patient. For example direct intramuscular injection of recombinant AAV vectors carrying

the beta-galactosidase gene in to muscle has resulted in persistent expression of the bacterial gene for 240 days (Fisher, K.J., et al., Nature Medicine, 1997, 3, 306-312)

In vivo Non-Viral Delivery of an Insulin gene into Patients

The non-viral insulin gene constructs can also be targeted in vivo to specific tissues, for example the muscle in patients. Examples of such delivery systems include liposome encapsulation, and direct injection of non-viral expression vectors.

(a) Liposome Encapsulation

Successful *in vivo* gene transfer has been achieved with the injection of DNA encapsulated in liposomes (Reviewed in Ledley, F.D., Human Gene Therapy, 1995, 6, 1129-1144; Farhood, et al., Ann NY Acad Sci, 1994, 716, 23-35). A number of cationic amphiphiles are now in developement (Ledley, F.D., Human Gene Therapy, 1995, 6, 1129-1144; Farhood, et al., Ann NY Acad Sci, 1994, 716, 23-35). Their low toxicity and efficacy in gene transfer in mice, after intravenous administration suggest that they are viable delivery systems for gene therapy (Stewart, et al., Human Gene Therapy, 1992, 3, 267-275; Nabel, et al., Human Gene Therapy, 1992, 3, 649-656). The expression cassettes for insulin gene in linear, plasmid or viral DNA forms may be condensed through ionic interactions with the cationic lipid to form a particulate complex for *in vivo* delivery (Stewart, et al., Human Gene Therapy, 1992, 3, 267-275).

Direct Injection

Direct injection of DNA segments comprising of an insulin gene into muscle either as a solution or as a calcium precipitate (Wolff, et al., Science, 1990, 247, 1465-1468; Ascadi, et al. The New Biologist, 1991, 3, 71-81; Benvenisty, et al., Proc Natl Acad Sci USA., 1986, 83, 9551-9555), provides for alternative technology for delivering the insulin gene expression cassette into muscle of patients.

Delivery of Glucokinase gene into cell, tissues and organs

(a) In vitro-Transfection of cells with plasmid constructs:

Recombinant DNA expression cassettes comprising of cellular promoters/enhamcers and regulatory regions operably linked to the glucokinase genes/cDNAs designed for expression in target mammalian tissues in the form of plasmids, linearized DNA fragments or viral DNA/RNA vectors are prepared and purified as described in Sambrook, et al., Molecular Cloning- A Laboratory Manual, CSHL, CSH, 1989.

Cells and cell lines to be transfected are grown in the recommended media (American Tissue Type Culture collection, Rockville, MD) supplemented with the appropriate growth factors, serum and antibiotics (Gibco/BRL, Grand Island, NY).

The DNAs are introduced into cells by one of the following methods, Calcium phosphate precipitation, DEAE-Dextran method, Electroporation (Ausudel, et al., Current Protocols in Molecular Biology, 1987, Wiley-Interscience), lipofectin (Ausudel, et al., Current Protocols in Molecular Biology, 1987, Wiley-Interscience) and protoplast fusion (Sandra-Goldin, et al., MCB., 1981, 1, 743-752). In the case where the selectable marker is on a separate plasmid, the Calcium phosphate co-precipitation method (Ausudel, et al., Current Protocols in Molecular Biology, 1987, Wiley-Interscience) is used.

24 hours after DNA-mediated transfection, the cells in culture are trypsinized and replated in selection media at a density of 1/10. Clonal cell line that have inherited the selectable marker are picked by ring cloning, expanded in culture and analyzed for the inheritance of the transfected gene of interest by PCR (Innis, et al., PCR Protocols: A guide to methods and applications, Academic Press, 1990) and Southern blot analysis (Southern, J Mol Biol, 1975, 98, 503) of genomic DNA prepared from the clonal cells/ cell lines. Expression of the transfected insulin gene are examined by Northern blot analysis (Sambrook, et al., Molecular Cloning- A Laboratory Manual, CSHL, CSH, 1989) of total RNA, by RIA analysis (CIS Biointernational, Gif-sur-Yvette, France) and by insulin activity assay (Gros, L et al. al., 1999, Human Gene Therapy, 10: 1207-1217).

Ex vivo Delivery of an Insulin gene

(a) In vitro Transduction of Cells in Culture

Primary muscle cells or myoblast are grown in the recommended media supplemented with the appropriate growth factors, serum, and antibiotics. The cells are transduced with the insulin gene either by direct infection with a recombinant viral vecotr described above or a non-viral delivery means, e.g. DNA mediated transfection. Recombinant DNA expression cassettes of muscle promoters/enhancers operably linked to the insulin genes/cDNAs designed for expression in muscle in the form of plasmids, linearized DNA fragments, or viral DNA/RNA vectors are prepared and purified as described in Sambrook, et al., Molecular Cloning- A laboratory Manual (Cold Spring Harbor Laboratory, 1989). DNA can be introduced into cells by DNA mediated transduction using one of the following methods: calcium phosphate precipitation, DEAE-Dextran method, electroporation (Ausudel, et al., Current Protocols in Molecular Biology (Wiley-Interscience, 1987), or lipofectin or protoplast fusion (Sandra-Goldin, et al., Mol Cell Biol., 1981, 1, 743-752). Where the selectable marker is on a separate plasmid, the calcium phosphate co-precipitation method (Ausudel, et al., Current Protocols in Molecular Biology (Wiley-Interscience, 1987) is used.

(b) Ex vivo modification of cells for implantation

The technologies for virus and DNA-mediated gene transduction into mammalian cells (e.g., primary cells and cell lines) allows for ex vivo cellular engineering. These engineered cells can then serve as metabolic factories (Newgard, C.B., Diabetes, 1994, 43, 341-350; Hughes, et al., PNAS USA., 1992, 89, 688-692; Newgard, C.B., J Lab Clin Med., 1993, 122, 356-363), as novel drug delivery systems (Kasid, et al., PNAS USA., 1990, 87, 473-477;Chen, et al., Human Gene Therapy, 1995, 6, 917-926), as serrogate tissues or organs (Mendell, J.R., et al., N Engl J Med., 1995, 333, 832-838; Rhim, et al., Science, 1994, 263, 1149-1152), and as neo-organs (Thompson, et al., PNAS USA., 1989, 86, 7928-7932; Culliton, Science, 1989, 246, 747-749) upon appropriate implantation into the host.

For example, hepatocytes may be isolated from the liver (Ponder, et al., PNAS USA, 1991, 1217-1221; Pages, et al., Human Gene Therapy, 1995, 6, 21-30), commited to short term culture (Pages, et al., Human Gene Therapy, 1995, 6, 21-30), and then transduced with a viral or plasmid vector carrying the expression cassette comprising of the glucokinase cDNA under the transcriptional control of a liver specific promoter. The genetically modified hepatocytes are then harvested and transplanted into a recipient either via infusion of the cells into the portal vein (Wilson, et al., PNAS USA., 1990, 87, 6437-8441) or introduced intrasplenically (Ponder, et al., PNAS USA, 1991, 1217-1221). The genetically modified hepatocytes introduced intrasplenically were shown to replace up to 80% of the diseased liver (Rhim, et al., Science, 1994, 263, 1149-1152). In dogs, a 5% replacement of the liver mass with hepatocytes transduced with the human α1-antitrypsin expressing retrovirus resulted in the expression of the human peptide for up to 30 days (Kay, et al., PNAS USA, 1992, 89, 89-93). Similarly, hypercholesterolemia in Watanabe heritable hyperlipidemic rabbits were transiently corrected by implantation of hepatocytes transduced with a retrovirus capable of directing the expression of a functional LDL receptor (Wilson, PNAS USA, 1990, 87, 8437-8441). While the vectors used in these experiments relied on the viral promoters for transcriptional control (which have a tendency to be turned off in vivo), it is anticipated that an internal liver specific promoter could produce a sustained level of expression of the transgene.

In another example, myoblasts may be isolated from muscle biopsies (Mendell, et al., N Engl J Med, 1995, 832-838) , expanded in culture and genetically modified to express high levels of insulin by transfection with DNA comprising of the insulin gene under the transcription control of a strong muscle specific promoter/enhancer or infected with a muscle specific recombinant retrovirus (Ferrari, et al., Human Gene Therapy, 1995, 6, 733-742).

The insulin expressing myoblasts can then be transferred into muscle my direct injection of the cells. Previous experience with murine myoblast have demonstrated that the injected myoblasts will fuse into preexisting multinucleate myofibrils (Dhawan, et al., Science, 1991, 254,1509-1512; Barr & Leiden, Science, 1991, 254, 1507-1509) and the differentiated muscle fibers will maintain a high level of expression of the transgene (Yao & Kurachi, PNAS USA., 1992, 89, 3357-3361; Bohl, et al., Nature Medicine, 1997, 3, 299-305).

The cells are then embedded into collagen coated lattices of expanded polytetrafluoroethylene (Gore-Tex) fibers as previously described (Thompson, et al., PNAS USA., 1989, 86, 7928-7932; Moullier, et al., Nature Genetics, 1993, 4, 154-159). Adsorption of of heparin-binding growth factors 1 to the collagen lattices, upon implantation into the peritoneal cavity will induce vascularization and formation of a neo-organ. Such neo-organs were reported to produce a sustained expression of trangenes in mice (Salvetti, et al., Human Gene Therapy, 1995, 6, 1153-1159) and dogs (Moullier, et al., Nature Med., 1995, 1, 353-357). It is therefore anticipated that such neo organs comprising of fibroblast or other cell type overexpressing insulin could potentially serve to normalized blood sugars in the diabetic state.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Also, all publications, patent applications, patents, and other references mentioned herein are incorporated by reference.

Examples

The following are examples which demonstrates certain aspect of how to practice the therapeutic process of this invention. It is believed that one skilled in the art, based upon the description herein, can utilize the invention to its fullest extent. The specific examples set forth below are, therefore to be construed as merely illustrative and not limitative of the remainder of the disclosure whatsoever.

Example 1. Construction of the MLC-mutated insulin insulin chimeric gene

The human cDNA encoding proinsulin containing the furin consensus cleavage site mutations was obtained by EcoR1 digestion of pP2.4-Insm (Gros, et al., Human gene Therapy, 1997, 8, 2249-2259). The cDNA was inserted into the EcoR1 unique site of a plasmid carrying a 1.3kb rat MLC1 promoter regulatory sequence along with 800bp of polyadenylation and splice signals of the SV40 small tumor antigen, and an 800bp genomic fragment of the MLC1 gene containing a strong muscle-specific enhancer known

to enhance the muscle-specific expression (Donoghue, et al., Gene Dev., 1988, 2, 1779-1790). The plasmid was designated pMLC/Insm (Figure 1).

Example 2. Cell culture and Transfection

C2C12 mouse myoblast was grown in monolayers in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL, Grand Island, NY). The cells were maintained at 37°C under 95% air, 5% CO2 atmosphere. C2C12 cells were co-transfected by the calcium phosphate co-precipitation method with both the pMLC/Insm plasmid (10ug) and a pPGKneo plasmid(2ug) carrying the neomycin selectable marker. Cells expressing the neomycin-resistance gene were selected in media containing 1mg/ml of geneticin (G418, GibcoBRL, Grand Island, NY). The presence of the MLC/Insm chimeric gene was detected by PCR using oligonucleotides ol.1 (5'-GTCAAGCGAGCCACCACTC-3') AND ol.2 (5'-CGGCGGGTCTTGGGTGTG-3'), which amplified a specific 0.5kb band (Figure 1A). The positive clones were screened for expression of the proinsulin gene by Northern blot analysis.

Example 3. C2C12 differentiated muscle cells expressing a mutated human proinsulin gene

C2C12 myoblast cells were differentiated in the presence of DMEM supplemented with 2%(v/v) horse serum (GibcoBRL, Grand Island, NY) for 4 days. RNA was isolated before and each day thereafter during the differentiation program by guanidinium isothiocyanate method (Chrigwin, et al., Biochemistry, 1979, 18, 5294-5299) and insulin gene expression was determined. RNA samples (30ug) were electrophoreses on 1% agarose gels containing 2.2M formaldehyde. Northern blots were hybridized to the following 32P-labeled probes: a 0.4kb EcoR1-EcoR1 fragment corresponding to the rabbit P-enolpyruvate carboxykinase (PEPCK) cDNA; a 1.3kb EcoR1-EcoR1 fragment corresponding to the rabbit ß-actin cDNA; and a 1.1kb EcoR1-EcoR1 fragment corresponding to the mouse myogenin cDNA. The probes were labeled using [α-32P]dCTP, following the random oligopriming method (Boehringer Mannheim, Germany). Probed filters were placed in contact with Kodak XAR-5 films. The ß-actin signal (internal control) was used to correct for RNA loading differences. Densitometric analysis of autoradiograms was performed at non-saturating exposures with a scanning densitometer (Fujix, BAS 1000).

The expression of the myogenin was used as a marker of differentiation. Myogenin was detected from the first day of differentiation with a maximal expression on day 3 (Figure 1B). A similar pattern of expression was observed with the insulin probe indicating that the clonal line C2C12Insm expressed the chimeric insulin gene from day 1-

4 of differentiation. No insulin gene expression was detected in control cells C2C12-neo after 4 days of differentiation. The chimeric insulin gene was not expressed in C2C12Insm in the undifferentiated state (day 0) (Figure 1 B). Differentiated C2C12Insm cells maintained their expression of insulin after one month in culture in the presence of 2% horse serum. These results indicate that with the MLC/Insm construct, differentiation of the C2C12Insm cells resulted in an induction and maintenance of the insulin gene expression in myotubes.

Example 4. Detection of insulin polypeptide production in C2C12 cells transfected with pMLC/Insm

Control and insulin-expressing C2C12 cells were grown on culture chamber slides (Nunc, USA). After one day of differentiation, the cells were washed in PBS and fixed in 4% paraformaldehyde and 0.05% glutaraldehyde for 20 min. The cells were then treated with 50mMNH4Cl for 20 min to quench any free aldehyde and then permeablized with 0.05% saponine for 15 min. Non-specific binding was saturated with 0.02% non-immune goat serum in PBS containing 1% bovine serum albumin (BSA). Cells were then incubated at room temperature for 1 hr with a mouse monoclonal anti-human insulin antibody (Sigma, St. Louis, USA) at 1/250 dilution. They were then washed in PBS and incubates at room temperature for 30 min with fluorescein-conjugated goat anti-mouse IgG (Boehringer Mannheim, Germany), at 1/50 dilution. The slides were examined for immunofluorescence using a confocal microscope (Leitz, Germany). The primary mouse anti-human insulin antibody specifically recognizes human insulin but does not discriminate between proinsulin and mature insulin.

No insulin signal was observed in untransfected C2C12 cells or C2C12 cells transfected with the neo-plasmid in the differentiated state (Figure 2A and 2 B). However, there was a strong insulin signal in the cytoplasm of all C2C12Insm differentiated cells (Figure 2C and 2D). The results confirm the expression of the human insulin polypeptide in the clones that have inherited the MLC/Insm gene construct.

Example 5. Analysis of proinsulin processing.

To evaluate the processing of proinsulin, C2C12-neo and C2C12Insm cells were incubated in the presence of 2% horse serum. After 3 days of differentiation, the cells were cultured for 24 hr in serum free medium supplemented with 20mM glucose. Culture medium and cell extracts from the control C2C12neo and insulin expressing C2C12Insm cell lines were analysed by high pressure liquid chromatography (HPLC). Each fraction obtained form the HPLC separation was subjected to radioimmunoassay for insulin.

No insulin immunoreactive products were detected in cells extracts and the culture medium from the differentiated C2C12neo cells (Figure 3A and 3B). In contrast, the profile of insulin immunoreactive products released to the medium by differentiated C2C12Insm cells indicated clear expression of human insulin (Figure 3A). Two peaks of insulin immunoreactive material, corresponding to the mature insulin and the proinsulin polypeptide according to their elution times were present. The major peak comprising of 90% of the material from the culture medium was mature insulin (fraction6-8). The remaining minor peak (about 10%) corresponded to the proinsulin product, fraction 12-14) (Figure 3A). A similar profile was obtained when cell extracts from the differentiated C2C12Insm were analyzed by HPLC (Figure 3B). A major peak (80%) corresponding to the mature insulin and a minor peak (20%) corresponding to the proinsulin fraction were detected.

The results indicate that it was possible to produce insulin in muscle cells. Furthermore, the insulin produced is principally in the processed mature form persumably by the furin endopeptidase present in differentiated C2C12Insm cells. Thirdly, the mature insulin was secreted into the medium and the secreted form represented the major fraction produced by the cells.

Example 6. Quantitation of insulin production from the C2C12Insm cell line

After three days of differentiation, C2C12neo and C2C12Insm cells were cultured overnight in serum free medium supplemented with 20mM gluocse, washed and then incubated for the various time period in the same medium containing 0.5% bovine serum albumin (BSA) and 0.1mg/ml aprotinin (Protease inhibitor). Aliquots were taken from the culture medium and immunoreactive insulin was measured. The release of insulin was linear during the first three hours and corresponded to about 100uU/10^6 cells (Figure 4A). Thereafter insulin continued to accumulate in the medium and high levels were noted 48hr after culture in serum-free medium. As expected, no insulin was detected in the culture medium of the differentiated control C2C12neo cell line (Figure 4A).

Example 7. Long term production of insulin in C2C12Insm cell line

Insulin production by the C2C12Insm cells was also studies after long term differentiation. To this end, cells were cultured in DMEM supplemented with 2%(v/v) hrose serum for up to 42 days. Several days after differentiation, cells were washed three times with serum-free medium and incubated for 2 hr in serum free medium supplemented with 20mM glucose containing 0.5% BSA and 0.1mg/ml aprotinin. Afterwards, insulin production was measured in the incubation medium (Figure 4B) and used as a reference level of production. At any time period analyzed, C2C12Insm cells

were able to release levels of insulin comparable to that produced 3 days after differentiation (Figure 4A and 4B). Thus, C2C12Insm cells were able to maintain a relatively constant level of insulin production in long-term culture. The results indicated that the MLC1-promoter is able to direct constitutive expression of the chimeric human insulin gene in mouse myotubes.

Example 8. Activity of the secreted human insulin

To determine if the secreted insulin is functional, stimulation of glucose uptake, glucose utilization, glucose consumption and lactate production by the insulin produced were evaluated in C2C12neo and C2C12Insm differentiated cells. After three days of differentiation, C2C12neo and C2C12Insm were cultured overnight in serum-free medium supplemented with 20mM glucose, washed and then incubated for variuos time period in the same medium containing 0.5%BSA and 0.1% aprotinin.

Under this condition, differentiated C2C12Insm cells showed a 2.5-fold increase in glucose uptake and consumption and a 5-fold increase in lactate production compared to the control C2C12neo cells (Figure 5A and 5B). The results indicated that insulin production by the differentiated C2C12Insm cells is functional and induced glucose uptake and utilization in the cells.

In another measure of activity, the biological activity of the secreted insulin was analyzed in FTO-2B rat hepatoma cells in culture. FTO-2B cells were incubated for 2 hours in the presence of media from C1C12Insm and C2C12neo control cells. At the end of that period, total RNA was obtained from the FTO-2B cells and the expression of an endogenous insulin responsive PEPCK gene (expression suppressed by insulin) was analyzed. Figure 6A and 6B is a Northern blot analysis using the PEPCK gene fragment as a probe. The results showed a marked reduction in the expression of PEPCK gene in FTO-2B cells exposed to media obtained from the differentiated C2C12Insm for 2 hr. In contrast, no decrease in PEPCK gene was observed in FTO-2B cells cultured in media from the control C2C12neo cells. The level of suppression in the former case was equivalent to that observed for FTO-2B cells treated with $\mathbf{10^{-7}M}$ of exogenous insulin (Figure 6A and 6B). The results indicated that the genetically engineered C2C12Insm cells are able to produce mature, biologically active insulin comparable to that of ß-islet cells in a sustained manner.

Example 9. Transplantation of C2C12Insm myoblast into skeletal muscle of diabetes mice.

To determine whether differentiated C2C12Insm cells were able to produce insulin in vivo, C2C12Insm myoblast cells were transplanted by direct skeletal muscle injection to

syngeneic C3H mice made diabetic by Streptozotocin (STZ) treatment. Intraperitoneal injection of 40mg of STZ (Sigma, St Louis, MO) into 3-weeks old C3H mice for 5 consecutive days led to a decrease of plasma insulin (from 125 ± 14 uU/ml to 60 ± 5 uU/ml) levels with a resultant development of hyperglycemia (from 132 ± 15 mg/dl to 340 ± 40 mg/dl) one week after the last STZ injection, assessed by measuring blood glucose levels. Diabetic mice were transplanted with either 2 x 10^6 control C2C12neo cells or 2 x 10^6 C2C12Insm myoblast cells by direct injection into the muscles of the two hindlimbs of the 5-weeks old diabetic C3H mice. Blood glucose and plasma insulin was measured in the fed animals 3 weeks after the injection of the cells, Blood glucose was determined by using the Glucometer Elite (Bayer, Germany) according to manufacturers instructions. Insulin was measure in the serum by radioimmunoassay ((CIS Biointernational, Gif-Sur-Yvette, France).

One week after transplantation of the C2C12Insm myoblast cells in the diabetic C3H mice, a high increase in serum insulin levels was observed (Figure 7A). The concentration of insulin reached a level similar to that of non-STZ-treated control mice, which was about 3-fold higher that that of diabetic C3H animals transplanted with C2C12neo control cells (Figure 7A). The increase in serum insulin levels was maximal for up to 2-weeks after transplantation. Thereafter, insulin levels decreased. By 3-weeks after transplantation, the levels were still higher that that in diabetic animals transplanted with the control C2C12neo cells. The rise in serum insulin was paralleled with a corresponding decrease in blood glucose levels in the animals treated with the C2C12Insm cells. In addition, while three weeks after transplantation, mice treated with the control C2C12neo cells showed a very high levels of plasma glucose (>500mg/dl), i.e. severely diabetic, C2C12Insm transplanted mice maintained a significantly lower level of plasma glucose (290 ± 10 mg/dl), almost to normoglycemia (Figure 7B). The results suggest that C2C12Insm myoblast are able to survive, differentiate in vivo and produce biologically active insulin in a diabetic host after direct msucle transplantation.

We claim:

1. A method for treating diabetes mellitus, comprising the step of administering to a patient in need thereof a DNA segment, said DNA segment comprising an insulin gene and a promoter sequence, wherein said promoter sequence is operably linked to said insulin gene and wherein said DNA segment is effective for the expression of a therapeutically effective amount of insulin in said patient.

2. A method of claim 1, wherein said DNA segment is injected directly into a muscle of said patient.

3. A method of claim 1, wherein said promoter is a muscle-specific promoter.

4. A method of claim 3, wherein said promoter is MCL-1, MCL-2, MLC-3, Creatinine kinase, Myo-D, or myogenin.

5. A method of claim 2, wherein said DNA segment is an integral part of a vector.

6. A method of claim 2, wherein said DNA segment is in a pharmaceutical preparation.

7. A method of claim 5, wherein said vector is a viral vector.

8. A method of claim 7, wherein said vector is a retroviral vector, an adenoviral vector, an adeno-associated viral vector, a sindbis viral vector, or a herpes viral vector.

9. A method of claim 6, wherein said pharmaceutical preparation is a DNA ligand conjugate, a liposome or a calcium phosphate precipitate.

10. A method of claim 1, wherein said DNA segment is introduced to said patient in cells, said cells having being treated in vitro to incorporate said DNA segment.

11. A method of claim 10, wherein said cells are muscle cells.

12. A method of claim 11, wherein said muscle cell is mouse myoblast C2C12, C3H10T1/2, rat myoblast L6, rat myoblast L8, human myoblast HISM, or a primary human myoblast.

13. A method of claim 10, wherein said cells are directly injected into a muscle of said patient.

14. A method of claim 10, wherein said cells are in a neo-organ.

15. A method of claim 10, wherein said cells are in a alginate microcapsule.

16. A method of claim 10, wherein said cells are in an immuno-privileged cell factory.

17. A method of claim 10, wherein said DNA fragment has been incorporated into said cells by DNA-mediated transfection.

18. A method of claim 10, wherein said DNA fragment has been incorporated into said cells by viral infection.

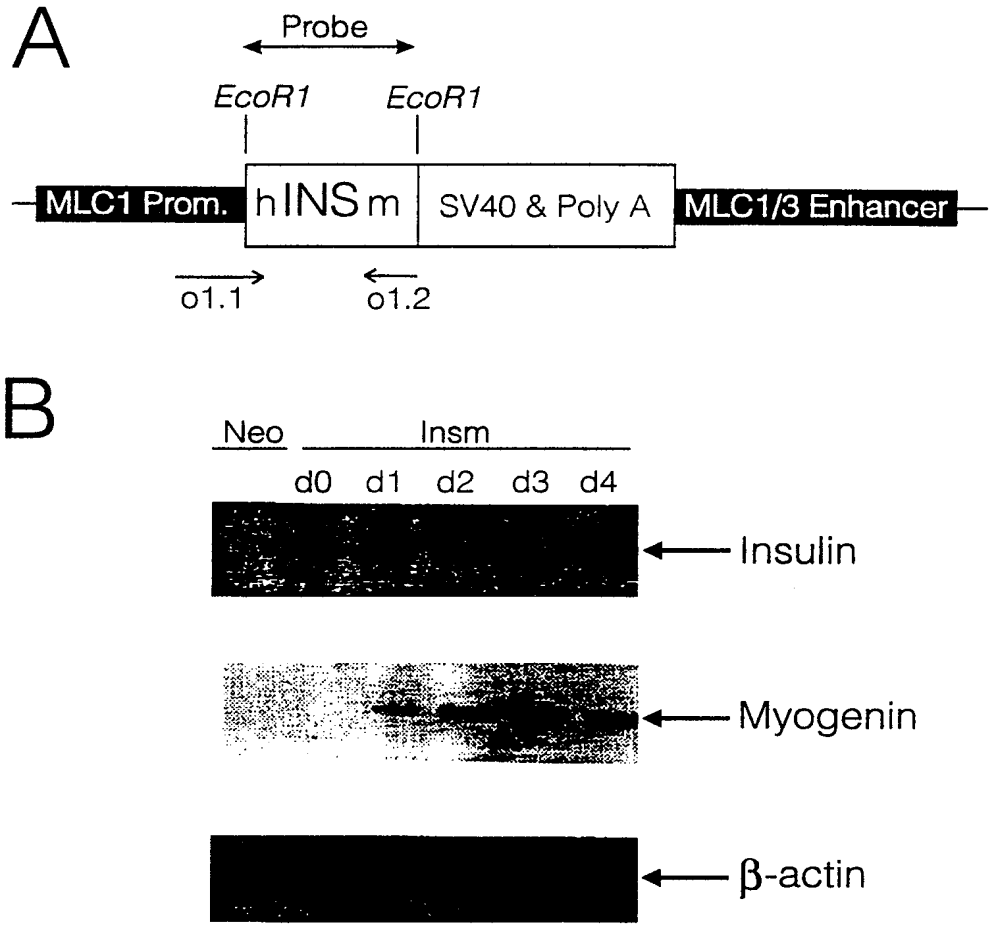

A
Probe
EcoR1
EcoR1
MLC1 Prom.
hINSm
SV40 & Poly A
MLC1/3 Enhancer
o1.1
o1.2
B
Neo
Insm
d0 d1 d2 d3 d4
Insulin
Myogenin
β-actin

Fig. 1

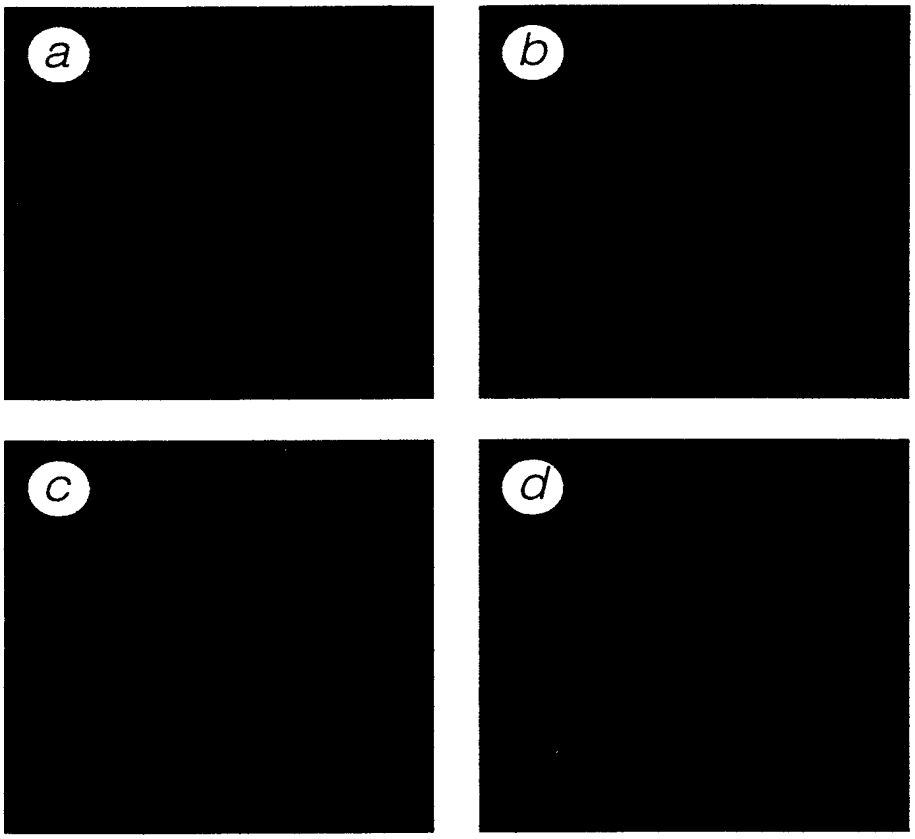


Fig. 2

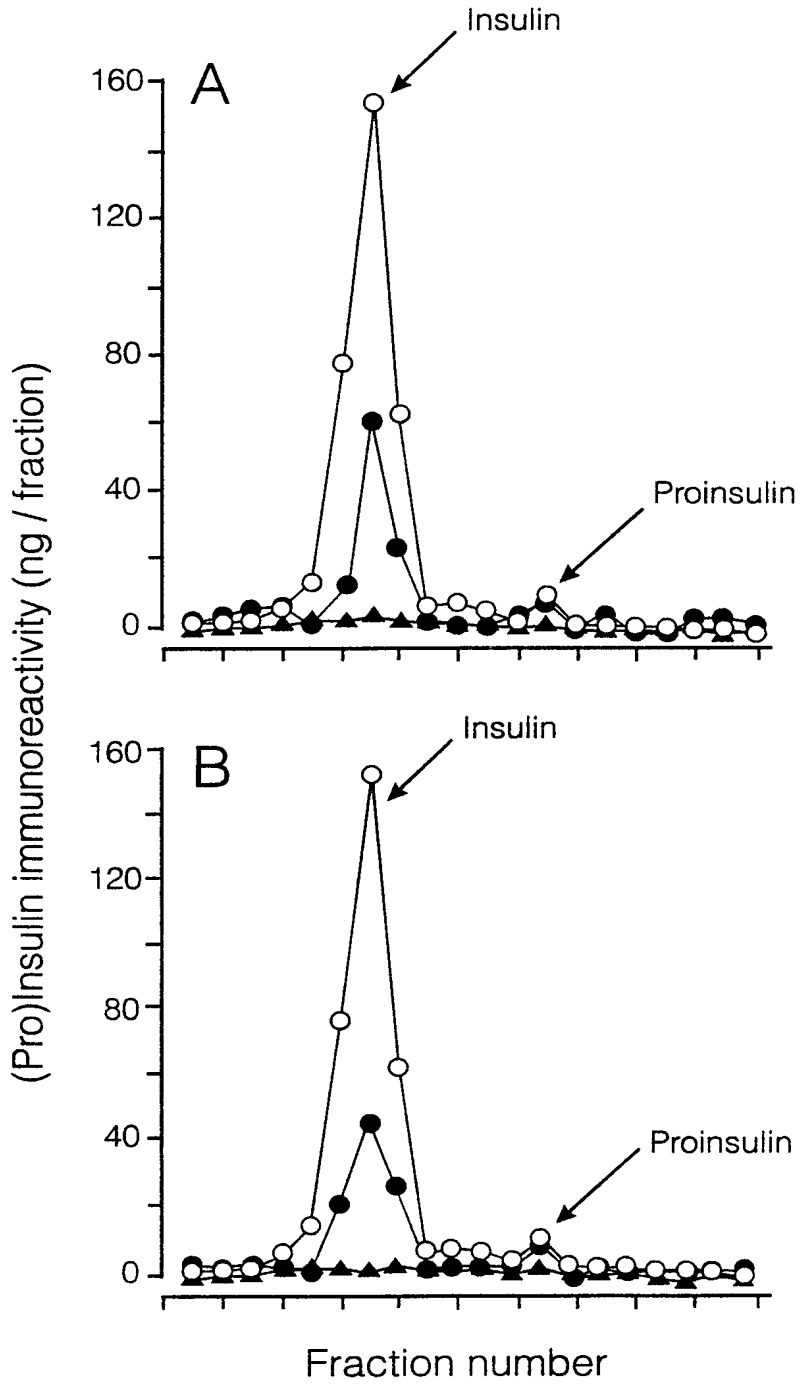

Insulin
A
160
120
80
40
0
Proinsulin
Insulin
B
160
120
80
40
0
Proinsulin
(Pro)Insulin immunoreactivity (ng / fraction)
Fraction number

Fig. 3

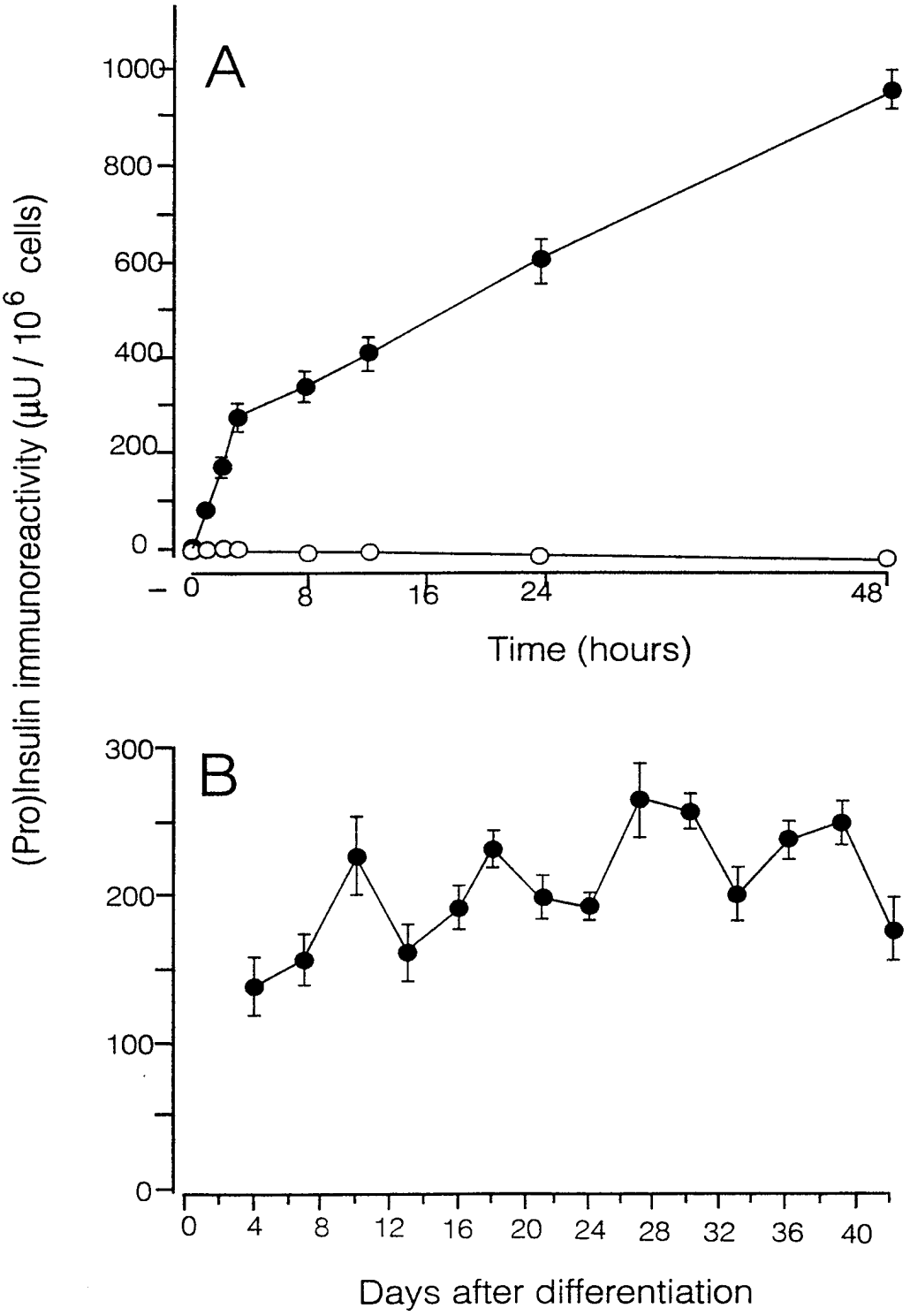

A
1000
800
600
400
200
0
0
8
16
24
48
Time (hours)
(Pro)Insulin immunoreactivity (µU / 10^6 cells)
B
300
200
100
0
0
4
8
12
16
20
24
28
32
36
40
Days after differentiation

Fig. 4

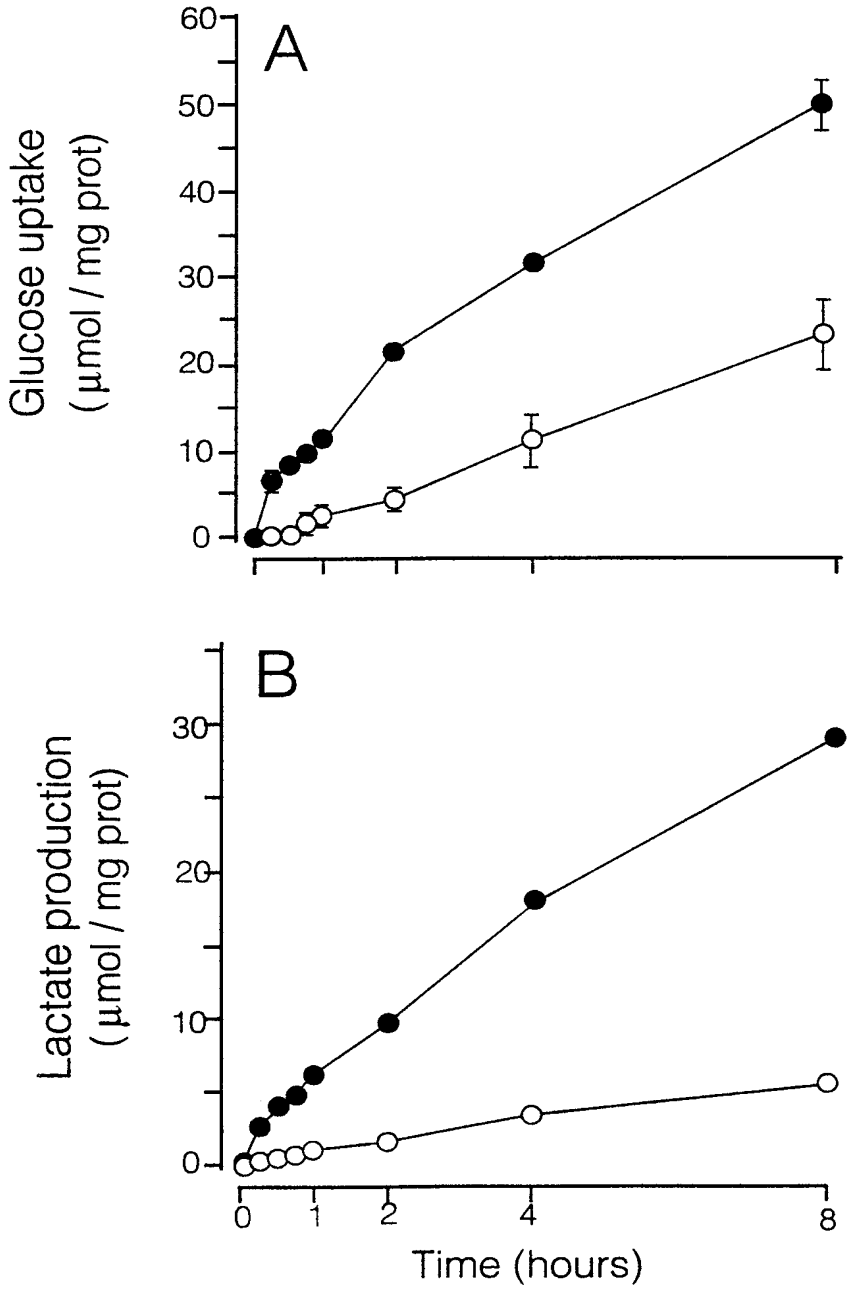

A
Glucose uptake
(µmol / mg prot)
60
50
40
30
20
10
0
B
Lactate production
(µmol / mg prot)
30
20
10
0
0
1
2
4
8
Time (hours)

Fig. 5

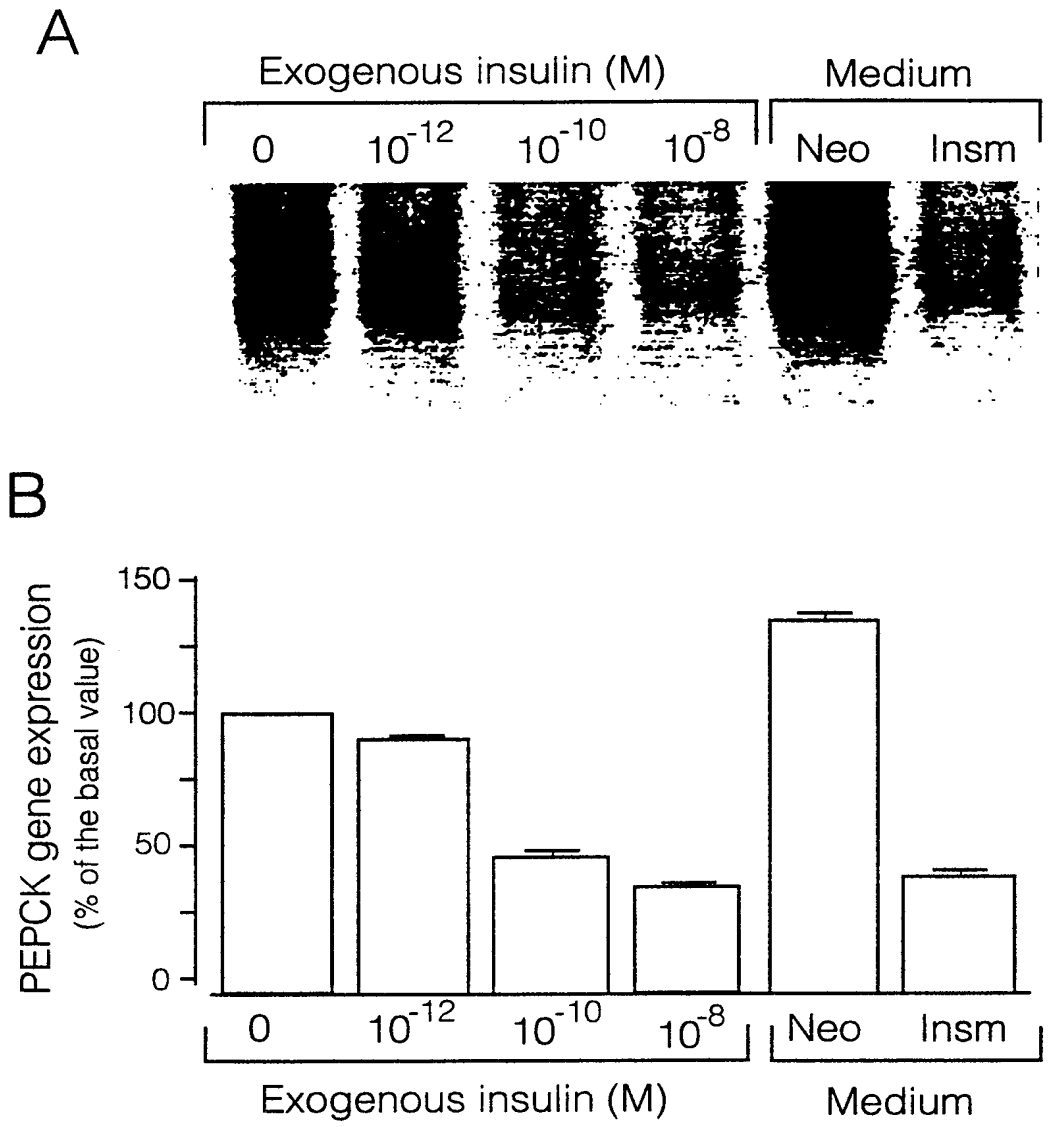

A
Exogenous insulin (M)
Medium
0
10-12
10-10
10-8
Neo
Insm
B
PEPCK gene expression
(% of the basal value)
150
100
50
0
0
10-12
10-10
10-8
Neo
Insm
Exogenous insulin (M)
Medium

Fig. 6

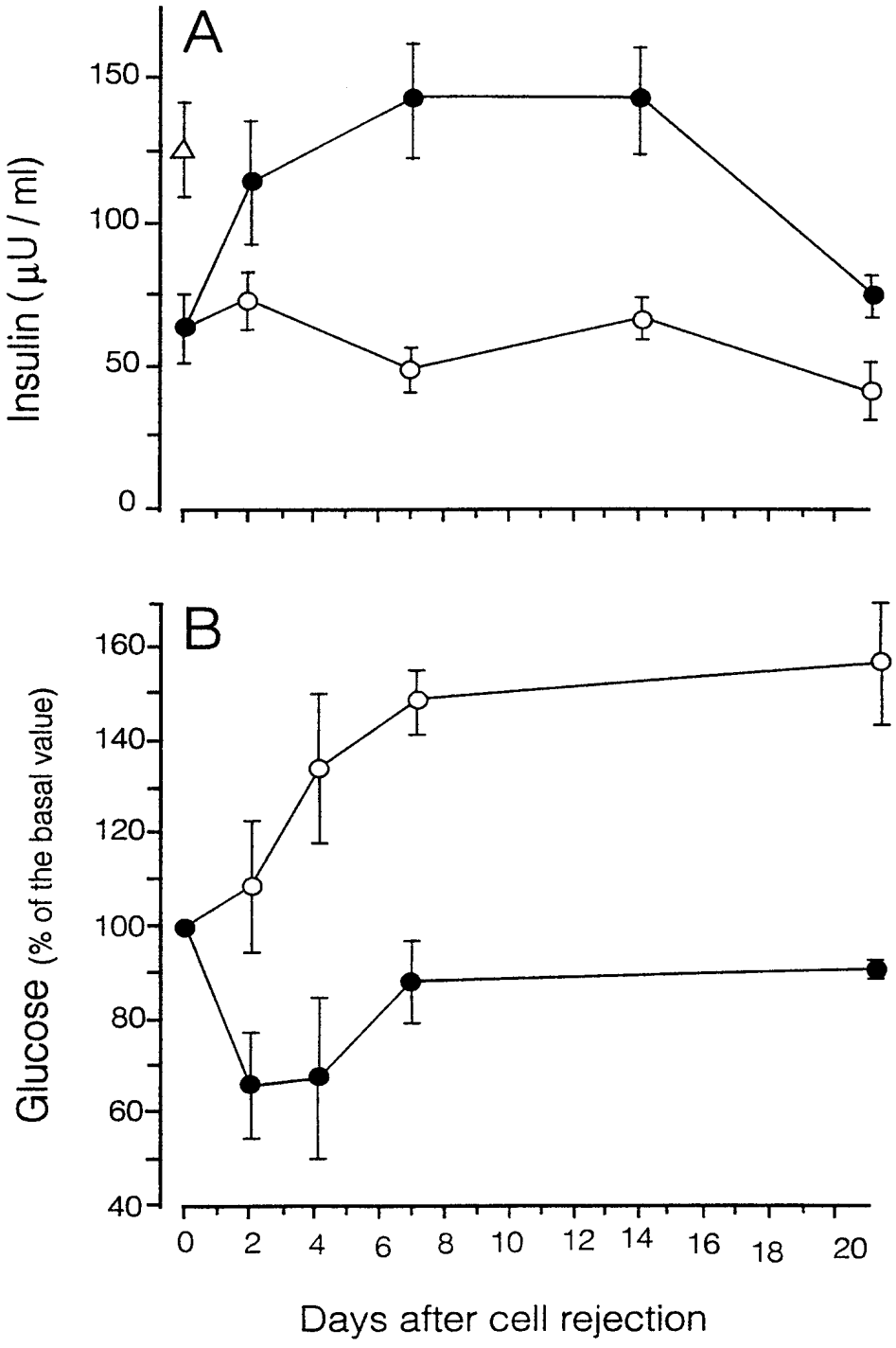

A
150
100
50
0
Insulin (µU / ml)
B
160
140
120
100
80
60
40
Glucose (% of the basal value)
0 2 4 6 8 10 12 14 16 18 20
Days after cell rejection

Fig. 7

INTERNATIONAL SEARCH REPORT

Int. onal Application No
PCT/EP 99/09132

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/17 A61K48/00 A61K35/34 A61P5/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GROS L ET AL: "Insulin production by engineered muscle cells." 34TH ANNUAL MEETING OF THE EUROPEAN ASSOCIATION FOR THE STUDY OF DIABETES;BARCELONA, SPAIN; SEPTEMBER 11, 1998, vol. 41, no. SUPPL. 1, August 1998 (1998-08), page A247 XP000892257 Diabetologia Aug., 1998 ISSN: 0012-186X abstract --- -/--	1-18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 2000	05/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Niemann, F

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	BARTLETT ET AL: "toward engineering skeletal muscle to release peptide hormone from the human pre-proinsulin gene" TRANSPLANT PROC, vol. 30, no. 2, 1998, page 451 XP000900065 the whole document	1-6
X	SIMONSON ET AL: "synthesis and processing of genetically modified human proinsulin by rat myoblast primary cultures" HUM GEN THER, vol. 7, no. 1, 1996, pages 71-78, XP000900066 abstract page 72, right-hand column, line 51 -page 73, left-hand column, line 7	1,2,5,6, 9
A	WO 97 26337 A (AVIGEN INC ;UNIV JOHNS HOPKINS (US)) 24 July 1997 (1997-07-24) abstract page 6, line 9 -page 7, line 6 page 21, line 1 - line 15	1-18
A	WO 98 07878 A (ARCH DEV CORP ;LEIDEN JEFFERY (US)) 26 February 1998 (1998-02-26) page 4, line 11 - line 23 page 5, line 10 - line 19 page 11, line 26 -page 12, line 2 page 12, line 15 - line 25 claims 1-9,15,16	1-18
A	MOULLIER P ET AL: "Long-term delivery of a lysosomal enzyme by genetically modified fibroblasts in dogs" NATURE MEDICINE,US,NATURE PUBLISHING, CO, vol. 1, no. 4, April 1995 (1995-04), pages 353-357, XP002115392 ISSN: 1078-8956 cited in the application the whole document	14
A	WO 93 03710 A (UNIV LEICESTER) 4 March 1993 (1993-03-04) the whole document	15

-/--

Inte nal Application No
PCT/EP 99/09132

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DEGLON N ET AL: "CENTRAL NERVOUS SYSTEM DELIVERY OF RECOMBINANT CILIARY NEUROTROPHIC FACTOR BY POLYMER ENCAPSULATED DIFFERENTIATED C2C12 MYOBLASTS" HUMAN GENE THERAPY, vol. 7, no. 17, 10 November 1996 (1996-11-10), pages 2135-2146-2146, XP000862853 ISSN: 1043-0342 cited in the application the whole document	15
A	WO 96 33264 A (UNIV SOUTH FLORIDA) 24 October 1996 (1996-10-24) the whole document	16
P,X	GROS LAURENT ET AL: "Insulin production by engineered muscle cells." HUMAN GENE THERAPY MAY 1, 1999, vol. 10, no. 7, 1 May 1999 (1999-05-01), pages 1207-1217, XP000900067 ISSN: 1043-0342 the whole document	1-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/09132

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-18
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. onal Application No
PCT/EP 99/09132

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9726337 A	24-07-1997	US 5858351 A	12-01-1999
		US 5962313 A	05-10-1999
		CA 2243261 A	24-07-1997
		CA 2243470 A	24-07-1997
		EP 0934411 A	11-08-1999
		EP 0874904 A	04-11-1998
		WO 9726336 A	24-07-1999
		US 5846528 A	08-12-1998
WO 9807878 A	26-02-1998	AU 4156697 A	06-03-1998
		EP 0874909 A	04-11-1998
WO 9303710 A	04-03-1993	AT 152616 T	15-05-1997
		AU 2443392 A	16-03-1993
		CA 2115664 A	04-03-1993
		DE 69219606 D	12-06-1997
		DE 69219606 T	04-09-1997
		DK 599942 T	03-11-1997
		EP 0599942 A	08-06-1994
		ES 2101863 T	16-07-1997
		GR 3024007 T	31-10-1997
		IL 102785 A	15-06-1998
		JP 6510035 T	10-11-1994
		MX 9204831 A	01-07-1993
		US 5529913 A	25-06-1996
		ZA 9206228 A	02-03-1993
WO 9633264 A	24-10-1996	AU 5390696 A	07-11-1996
		US 5827736 A	27-10-1998